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<b>13. ABSTRACT (Maximum 400 words)</b>  The effect of liquid gun propellant (LP) on the barrier function of skin was assessed <u>in vitro</u> by penetration of <sup>14</sup> C-benzoic acid and histopathology. Weanling pigs were topically exposed to 25 $\mu$ l/cm <sup>2</sup> of test compound [saline (control) or LP] for 1-5 days. Pigs were killed and skin sections, excised from the sites of application, were mounted on <u>in vitro</u> penetration chambers to measure cumulative 24-h penetration of <sup>14</sup> C-benzoic acid. Topical exposure to LP for 1, 2, 3, 4, and 5 days resulted in 8.2, 4.5, 2.8, 1.2 and 1.7-fold increases in permeability for <sup>14</sup> C-benzoic acid, respectively. Visual inspection of the LP-treated sites indicated that the macroscopic lesions (erythema and pustules) were most severe on day 3. The microscopic lesions observed in the LP-treated skin (Superficial perivascular lymphoplasmacytic dermatitis, edema and increased eosinophils) appeared to be independent of duration of LP exposure. The utility of this system was demonstrated in studies which showed that hydroxylamine does not appear to be the LP component responsible for the severe irritant properties of LP. <u>In vivo</u> topical (continued on back)				
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exposure of pigs to hydroxylamine HCL (HAH) for 1 day resulted in a 2.1-fold increase in skin permeability to  $^{14}\text{C}$ -benzoic acid. These studies demonstrated that the effect of LP on skin barrier properties is greatest at 1 day of exposure and steadily diminishes during subsequent exposure. This technique can be used to assess the effect of chemical or physical agents on barrier function and repair processes of skin.

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*Barbara W. Kempainen*

February 24, 1992

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## INTRODUCTION

Liquid Gun Propellant (LP), a mixture of 60.8% hydroxyl ammonium nitrate, 19.2% triethanol ammonium nitrate and 20% water, can penetrate skin and cause systemic effects, the most prominent of which is the formation of methemoglobin (Parmer et al., 1992). In vitro studies (Reifenrath and Hawkins, 1990) demonstrated that damage to the epidermis greatly increases the penetration of a component(s) of LP which causes methemoglobin formation. There is concern that LP may degrade the protective properties of the epidermis following prolonged exposure and may therefore enhance its own penetration.

The purpose of the first phase of this study was to assess the barrier properties of pig skin following 1, 2, 3, 4, and 5 days of exposure to LP. The pig was chosen as an animal model because its skin is comparable in permeability to human skin (Reifenrath et al., 1984). In vitro measurements of percutaneous penetration of benzoic acid (Hawkins and Reifenrath, 1986) was used to assess changes in barrier function. In addition, exposure sites were biopsied at the various time intervals after exposure and histological examination (H & E stain) was used to evaluate damage to the epidermis and dermis.

The purpose of the second phase of this study was to determine if hydroxylamine is the primary component of LP responsible for the degradation of the barrier properties of skin caused by LP. Hydroxylamine is a skin irritant (Gross, 1984).

## BODY

### Materials and Methods

*Phase 1:* Six weanling cross-bred Yorkshire pigs (Gullatte Farm, Salem, AL) weighing 21 to 25 kg were used for this study. Hair from the upper back of the animals was removed with electric clippers (Oster, Milwaukee, WI). The upper back of each pig had six application sites (three control sites were paired with three treated sites). Each application site had a surface area of 3 cm<sup>2</sup>. The control sites were dosed with 75 µl saline and the treated sites were dosed with 75 µl LP. The application of saline or LP to the paired sites was done according to the schedule in Table 1 (pigs 1-5). A blunt-tipped Hamilton<sup>R</sup> glass syringe was used to apply the dose to a layer of cotton gauze covering the application site. The cotton gauze became saturated with the dose and held the dose at the application site. A non-occlusive protective covering was then placed over the application site.

At the end of the exposure period, the protective coverings were removed and the gross appearance of the skin at the application sites noted. The epidermal surfaces were cleaned with cotton gauze moistened with water. The pigs were euthanized with an intramuscular injection of xylazine HCl (3 mg/kg, Rompun, Miles Laboratories, Shawnee, KS) and ketamine HCl (20 mg/kg, Vetalar, Parke-Davis, Morris Plains, NJ), followed by an intravenous injection of sodium pentobarbital (18 mg/kg, Anthony Products, Arcadia, CA). A Padgett Electro Dermatome (Padgett Instruments, Kansas City, MO) was used to remove sections of skin 900 µm thick at the site of application. A 6 mm biopsy punch was used to remove a portion of the skin from a peripheral area of the application site for histological examination. A disk of skin was cut from each application site for placement in flowing percutaneous penetration chambers (LGA #1083, Laboratory Glass Apparatus, Berkeley, CA). The skin disks were held in place between the donor and receptor chambers with a clamp. The dose (3.2 µg/0.1 µCi <sup>14</sup>C-benzoic acid [New England Nuclear, Boston, MA] dissolved in 5 µl of acetone) was applied to each epidermal surface (area - 0.8 cm<sup>2</sup>). The receptor fluid (RPMI media, GIBCO, Gaithersburg, MD) was pumped

through the receptor chambers of the diffusion apparatus at a rate of 4 ml/hour. A fraction collector was used to collect hourly aliquots of receptor fluid as it exited the diffusion chambers. Skin penetration of <sup>14</sup>C-benzoic acid was monitored for 24 hours.

At the end of the exposure period, the benzoic acid remaining on the epidermal surfaces was removed with cotton-tipped swabs moistened with alcohol, followed by dry swab. The center, dosed portion of the skin was cut away from the outer un-dosed portion of the skin. The center and outer portions of the skin were separately solubilized with Soluene (Packard, Downers Grove, IL). Radioactivity in the cotton swabs and solubilized skin was determined by adding scintillation cocktail (Ultima Gold, Packard) to each vial and counting on a liquid scintillation counter (Packard). Receptor fluid was combined with cocktail and radioactivity measured. Total recovery of dose was calculated by summing radioactivity in: receptor fluid, dosed portion of skin, un-dosed portion of skin, and surface wash.

*Phase 2:* Selection of exposure period and sample size to be used in Phase 2 was based on the results from Phase 1. The period of exposure to LP which resulted in the greatest decrease in the barrier properties of pig skin (1 day) was used for all groups in Phase 2. A sample size of 3 pigs was used since this was the sample size used in Phase 1 experiment.

The dosing regimen used in Phase 2 is shown in Table 1 (pigs 6-8). The doses applied were: (i) 132  $\mu$ l of hydroxylamine, (ii) 75  $\mu$ l LP [positive control], and (iii) 75  $\mu$ l saline [negative control].

The concentration of hydroxylamine hydrochloride (HAH) in water used in this study was based on the published composition of LP1846 (hydroxylammonium nitrate [HAN], triethanolammonium nitrate, and water in the ratio of 60.8:19.2:20 [v/v]). The dose of HAH applied was equivalent to the number of moles of hydroxylammonium nitrate in 75  $\mu$ l of LP. Due to limited solubility of HAH in water, each HAH dose was dissolved in 132  $\mu$ l of water (in contrast to 75  $\mu$ l of LP dosed).

Three weanling cross-bred Yorkshire pigs weighing 20 to 24 kg were used

for this study. Application sites were prepared and dosed as described above.

At the end of the exposure period the non-occlusive coverings were removed and gross appearance of application sites noted. The pigs were euthanized and skin sections removed from the application sites as described above. Histological examination and  $^{14}\text{C}$ -benzoic acid percutaneous penetration studies were also completed as described above.

Results.

Phase 1: The saline treated sites appeared normal. The lesions in the LP treated sites appeared to be most severe (intense erythema and 3 to 8 pustules with deep erosions of the skin in some cases) after 3 days of exposure. After 1 or 5 days of exposure to LP the lesions were minimal.

Microscopic lesions in LP treated skin included superficial perivascular lymphoplasmacytic dermatitis, mild edema in the papillary dermis, mild increase in the number of dermal eosinophils, intra-epidermal neutrophilic pustules associated with mild acanthosis and increased numbers of dermal neutrophils. The severity of the microscopic lesions appeared to be independent of time of exposure to LP. See Appendix 1 for pathology report.

The results from the percutaneous penetration experiments are shown in Table 2. The results represent data from 5 pigs which each had 3 control sites paired with 3 treated sites (period of exposure varied between 1 to 5 days).

The effect of LP on the barrier property of pig skin was calculated by dividing the radioactivity in the receptor fluid bathing skin dosed with LP by the radioactivity in the receptor fluid bathing the paired saline dosed control site. The mean plus or minus standard deviation was calculated from 3 paired comparisons. In pigs exposed to LP and saline for 1, 2, 3, 4, and 5 days: LP reduced the barrier properties of the skin by factors of  $8.2 \pm 3.4$ ,  $4.5 \pm 3.8$ ,  $2.8 \pm 1.4$ ,  $1.2 \pm 0.84$ ,  $1.7 \pm 0.26$ , respectively.

Phase 2: Gross appearance of application sites dosed with HAH were not distinguishable from the saline treated sites (both appeared normal). Histological examination of the application sites indicated HAH treated skin was indistinguishable from application sites treated with saline. Microscopic lesions in LP treated skin were similar to those observed in Phase 1 (see Appendix 2).

The effect of 1 day of exposure to the test chemicals (HAH, LP, or saline) on the barrier property of pig skin is shown in Table 3. The effect of the test chemicals on barrier properties was calculated by dividing the

:radioactivity ( $^{14}\text{C}$ -benzoic acid) in the receptor fluid bathing skin dosed with HAH or LP by the radioactivity in the receptor fluid bathing the paired control site (dosed with saline). The mean plus or minus the standard deviation was calculated from 3 or 4 replicates. Topical exposure to HAH or LP for 1 day resulted in  $2.07 \pm 1.26$  or  $10.50 \pm 13.00$  fold increase in percutaneous penetration of  $^{14}\text{C}$ -benzoic acid, respectively.

#### CONCLUSION AND DISCUSSION

LP altered the barrier property of the skin as evidenced by increased penetration of benzoic acid through the skin treated with LP compared to saline. The most dramatic effect of LP on the barrier property was after 1 day of exposure, and the effect gradually decreased as the exposure period to LP and saline was increased. The time course of the effects of LP on barrier properties is consistent with a previous report in which in vivo exposure to an irritant (sodium lauryl sulfate) decreased the barrier properties of hairless guinea pig skin to the greatest extent after 1 day, and the barrier returned to normal after 3 days exposure (Wilhelm et al., 1991).

The time course of the effect on percutaneous penetration did not correlate with the time course of gross or microscopic lesions at the site of application. The gross lesions appeared most severe after 3 days of exposure, while the lesions appeared much less severe after 1 or 5 days of exposure. We postulate the difference in time course is because the change in barrier properties is due to a direct corrosive effect of the LP on the stratum corneum which occurs immediately (minutes to hours) upon contact, while the gross lesions occur due to an inflammatory process which takes more time (days) to develop fully. Furthermore, the lack of correlation between the time course for development of microscopic lesions and the time of exposure to LP is probably related to obtaining the biopsy from a peripheral area of the application site. The central portion of the application site was consistently the most severely affected by the LP. It was necessary to collect the biopsy in this manner in order to prepare a sufficiently large skin disk for the skin penetration studies. In future studies, we recommend having separate application sites which will serve for histopathological evaluation and a separate (paired) application site which will serve for preparing skin disks for penetration studies.

The results from the Phase 2 experiment indicated that HAH does not contribute substantially to the gross or microscopic lesions caused by LP, nor to the decreased barrier property caused by LP. HAH was chosen to represent

HAH. It may be the salt form is critical. We propose that follow-up studies should be done to test if hydroxyl ammonium nitrate (HAN) is the component of LP responsible for lesions and decreased barrier properties of LP.

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Table 1. Experimental design for in vivo pretreatment of pigs with saline (control), Liquid Gun Propellant (LP), or Hydroxylamine HCl (HAH)

Pig	Site	Test Material	Dose Time	Pig	Site	Test Material	Dose Time
1	A	LP	day 0	2	A	LP	day 1
1	B	Saline	day 0	2	B	Saline	day 1
1	C	LP	day 1	2	C	LP	day 2
1	D	Saline	day 1	2	D	Saline	day 2
1	E	LP	day 2	2	E	LP	day 3
1	F	Saline	day 2	2	F	Saline	day 3
		Sacrifice	day 3			Sacrifice	day 4
3	A	LP	day 0	4	A	LP	day 1
3	B	Saline	day 0	4	B	Saline	day 1
3	C	LP	day 3	4	C	LP	day 2
3	D	Saline	day 3	4	D	Saline	day 2
3	E	LP	day 4	4	E	LP	day 3
3	F	Saline	day 4	4	F	Saline	day 3
		Sacrifice	day 5			Sacrifice	day 6
5	A	LP	day 2	6	A	LP	day 7
5	B	Saline	day 2	6	B	Saline	day 7
5	C	LP	day 3	6	C	HAH	day 7
5	D	Saline	day 3			Sacrifice	day 8
5	E	LP	day 6				
5	F	Saline	day 6				
		Sacrifice	day 7				
7	A	LP	day 8	8	A	LP	day 9
7	B	Saline	day 8	8	B	Saline	day 9
7	C	HAH	day 8	8	C	HAH	day 9
		Sacrifice	day 9			Sacrifice	day 10

Table 2. Effect of duration of exposure to liquid gun propellant (LP) on barrier property of pig skin (assessed by in vitro skin penetration by [<sup>14</sup>C] benzoic acid<sup>1</sup>)

DISPOSITION OF RADIO- ACTIVITY	Duration of Exposure									
	1 DAY		2 DAYS		3 DAYS		4 DAYS		5 DAYS	
	Control <sup>2</sup>	LP <sup>2</sup>	Control	LP	Control	LP	Control	LP	Control	LP
Receptor Fluid	0.97 $\pm$ 0.24	8.9 $\pm$ 6.1	3.3 $\pm$ 2.8	5.7 $\pm$ 1.8	1.8 $\pm$ 1.3	6.3 $\pm$ 0.5	2.0 $\pm$ 0.89	1.7 $\pm$ 0.65	2.6 $\pm$ 2.2	4.4 $\pm$ 3.4
Epidermal Surface	12 $\pm$ 9.7	5.0 $\pm$ 3.3	19 $\pm$ 10	6.0 $\pm$ 0.3	9.2 $\pm$ 7.0	14 $\pm$ 6.7	11 $\pm$ 5.9	9.0 $\pm$ 2.6	7.2 $\pm$ 3.2	15 $\pm$ 8.7
Skin Disk Exposed	61 $\pm$ 29	58 $\pm$ 11	54 $\pm$ 22	34 $\pm$ 4.1	51 $\pm$ 28	35 $\pm$ 10	58 $\pm$ 19	56 $\pm$ 16	55 $\pm$ 22	31 $\pm$ 22
Skin Disk Unexposed	25 $\pm$ 15	33 $\pm$ 46	37 $\pm$ 18	62 $\pm$ 9.6	32 $\pm$ 27	60 $\pm$ 15	33 $\pm$ 19	28 $\pm$ 19	25 $\pm$ 19	28 $\pm$ 15
Total Recovery	99 $\pm$ 19	105 $\pm$ 22	112 $\pm$ 4.1	108 $\pm$ 12	93 $\pm$ 30	115 $\pm$ 17	104 $\pm$ 6.6	95 $\pm$ 19	89 $\pm$ 15	78 $\pm$ 25

<sup>1</sup>Expressed as percent of dose of [<sup>14</sup>C] benzoic acid (0.1 $\mu$ Ci) applied topically to disks of excised pig skin

<sup>2</sup>Mean  $\pm$  standard deviation of three determinations done on three pigs

**Table 3. Effect of in vivo topical exposure\* to hydroxylamine hydrochloride and LP on barrier property of pig skin (assessed by in vitro skin penetration by [<sup>14</sup>C] benzoic acid<sup>1</sup>)**

Disposition of radioactivity	Treatment		
	Control: Saline	Control: LP	Hydroxylamine hydrochloride
Skin Penetration	0.87 ± 0.10	8.2 ± 7.7	1.8 ± 0.8
Skin Surface	29 ± 21	2.7 ± 0.69	8.7 ± 7.4
Skin Exposed	32 ± 8.3	41 ± 2.4	59 ± 13
Skin Unexposed	21 ± 14	33 ± 13	19 ± 17
Total Recovery	83 ± 16	85 ± 4.2	89 ± 18

\*Duration of exposure was 1 day for all treatment groups.

<sup>1</sup>Expressed as mean (± standard deviation) percent of dose of [<sup>14</sup>C] benzoic acid applied topically to disks of excised pig.

Appendix 1  
October 23, 1991

**The Effect of Liquid Gun Propellant on Porcine Skin**

**Histopathology**

Lesions in the skin from pigs treated topically with liquid gun propellant (LGP) included multifocal perivascular edema and superficial perivascular dermatitis of the papillary and periadnexal dermis. Severity varied from mild to moderate. The inflammatory cell infiltrate was composed of eosinophils, lymphocytes, few mast cells and occasional neutrophils. In mild lesions, the edema separated the pericapillary adventitia, and the inflammatory cells formed a thin cuff, around capillaries of the papillary dermis. In moderate lesions, the edema was more extensive and the inflammatory cell infiltrate was composed of increased numbers of cells. Edema and inflammation were observed around capillaries of the papillary and periadnexal dermis, extended for a variable distance into the dermis, and separated dermal collagen fibers.

The incidence and severity of lesions varied between pigs and between sites, and was independent of the number of LGP applications. Lesions were only observed in two pigs (pigs 4 and 5). Lesions of mild severity were observed in sites treated for 1 or 5 days (pig 4) and for 5 days (pig 5) with LGP. Lesions of moderate severity were observed in one site treated for 2 days (pig 4), and in one site treated for 3 days (pig 5) with LGP.

Appendix 2

The Effect of Liquid Gun Propellant on Porcine Skin

Histopathology - Group 2

Microscopic lesions were observed in skin of pigs treated topically with liquid gun propellant (LGP), with and without therapy (ascorbic acid). Treatment with ascorbic acid had no observable effect on the severity or distribution of microscopic dermal and epidermal lesions in LGP-treated skin. Severity of the microscopic lesions were independent of the duration of LGP or ascorbic acid treatment. No microscopic lesions were observed in skin treated topically with saline. Skin treated topically with hydroxylamine HCl was microscopically indistinguishable from sections of saline-treated skin.

In LGP-treated skin there was a multifocal, superficial, perivascular lymphoplasmacytic dermatitis with moderate perivascular infiltrate of eosinophils and few mast cells. Perivascular adventitia and dermal collagen fibers were separated by clear edema. Myofibers of the arrector pili muscles were vacuolated. Mild, focal acanthosis, basal cell hypertrophy and hyperplasia and hypergranulosis were observed in the epidermis. Two sections (one treated with LGP for three days and one treated with LGP plus ascorbic acid for one day) had one or two intraepidermal neutrophilic pustules and mild neutrophilic exocytosis.

Histopathology

**Bibliography of publications resulting from contract**

**Appendix 3**

1. Kemppainen, BW, Terse, P, Madhyastha, MS, Lenz, S, Palmer, W, and Reifenrath, WG. Development of an in vitro method to assess damage to the barrier properties of skin. Presented: Phi Zeta Research Emphasis Forum, Auburn University, February 17-20, 1992.
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**List of all personnel who received pay from contract No. DAMD17-91-C-1137**

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**Graduate degrees resulting from the contract support: None**